

10/517210

PCT/ SE 03 / 0 0 9 8 3

PRVPATENT- OCH REGISTRERINGSVERKET
Patentavdelningen**Intyg
Certificate**

REC'D 03 JUL 2003

WIPO

PCT

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.



(71) Sökande Cartela AB, Lund SE
Applicant (s)

(21) Patentansökningsnummer 0201831-5
Patent application number

(86) Ingivningsdatum 2002-06-14
Date of filing

Stockholm, 2003-06-24

För Patent- och registreringsverket
For the Patent- and Registration Office

Sonia André
Sonia André

Avgift
Fee

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

Ink. t. Patent- och reg. verkst

2002-06-14

Huvudföreläsning

MARKER FOR STEM CELLS AND ITS USE

TECHNICAL FIELD

- This invention relates to a marker for isolation and identification of
- 5 mammalian mesenchymal stem cells. Also included are methods and uses of such a marker as well as an enriched cellular population and a cellular composition comprising the enriched cellular composition.

BACKGROUND OF THE INVENTION

10 *Mesenchymal stem cells*

- The adult body houses so called stem cells that are capable of dividing many times while also giving rise to daughter cells with specific phenotypical characteristics. Several types of stem cells exist in the body including embryonic stem cells, haematopoietic stem cells and mesenchymal stem cells. Mesenchymal
- 15 stem cells are able to form mesenchymal tissues such as bone, cartilage, muscle, bone, ligament, fat and bone marrow stroma. Figure 1 shows a schedule of suggested stepwise transitions from putative mesenchymal stem cells (MSC) to highly differentiated phenotypes. The mesenchymal stem cells are located in bone marrow, around blood vessels, in fat, skin, muscle, bone and other tissues. Their
- 20 presence contributes to the reparative capacity of these tissues.

Medical use of MSC

- Currently, the medical use of MSC is to explore their potential in the regeneration of tissues that the body cannot naturally repair or regenerate when
- 25 challenged. For this, MSC are isolated, expanded in culture and stimulated to differentiate into connective tissues such as bone, cartilage, muscle, bone marrow stroma, tendon, fat and others. These tissue-engineered constructs can then be re-introduced into the human body to repair lost or damaged tissue. In another approach MSC can be directly stimulated *in vivo* to induce the formation of specific
- 30 tissues *in situ*.

Having defined MSC as potential "building blocks" for tissue engineering and transplantation, researchers are now searching for better ways to identify, isolate and characterize MSC.

35 *Alpha10*

A newly discovered collagen-binding integrin, alpha10beta1, includes the integrin subunit alpha10 (Camper *et al.*, (1998) J. Biol. Chem. 273:20383-20389). The integrin is expressed on chondrocytes and shows a M_r of 160 kDa after reduction when isolated from bovine chondrocytes by collagen type II affinity

BEST AVAILABLE COPY

purification.

Cloning and cDNA sequencing showed that it shares the general structure of other integrin alpha subunits. The predicted amino acid sequence consists of a 1167-amino acid mature protein, including a signal peptide (22 amino acids), a long
5 extracellular domain (1098 amino acids) a transmembrane domain (22 amino acids), and a short cytoplasmic domain (22 amino acids). In contrast to most alpha-integrin subunits, the cytoplasmic domain of alpha10 does not contain the conserved sequence KXGFF(R/K)R. Instead, the predicted amino acid sequence in alpha10 is KLGFFAH. It is suggested that the GFFKR motif in alpha-chains are important for
10 association of integrin subunits and for transport of the integrin to the plasma membrane (De Melker *et al.* (1997) Biochem. J. 328:529-537).

The extracellular part contains a 7-fold repeated sequence, an I-domain (199 amino acids) and three putative divalent cation-a binding site. The deduced amino acid sequence of alpha10 is 35% identical to the integrin subunit alpha2 and 37%
15 identical to the integrin subunit alpha1. Sequence analysis has revealed that the alpha10 subunit is most closely related to the I domain-containing α subunits with the highest identity to alpha1 (37%), alpha2 (35%) and alpha11 (42%).

Alpha11

20 The alpha11 integrin has recently been identified on cultured human foetal muscle cells. The cloning and characterisation revealed an I-domain containing, beta1-associated protein.

The open reading frame of the cDNA encodes a precursor of 1188 amino acids. The predicted mature protein of 1166 amino acids contains 7 conserved
25 FGGAP repeats, an I-domain with a MIDAS motif, a short transmembrane region and a unique cytoplasmic domain of 24 amino acids containing the sequence GFFRS.

Alpha11 contains three potential divalent cation binding sites in repeats 5-7. The presence of 22 inserted amino acids in the extracellular stalk portion (amino
30 acids 804-826) distinguishes the alpha11 integrin sequence further from other integrin alpha-chains.

Amino acid sequence comparisons reveal the highest identity (42%) with the alpha10 integrin chain. Immunoprecipitation with antibodies to the alpha11 integrin captured a 145 kDa protein, distinctly larger than the 140 kDa alpha2 integrin chain
35 when analysed by SDS-PAGE under non-reducing conditions.

Isolation and identification of MSC

The identification of MSC *in situ* is hampered by the fact that mono-specific and unique molecular probes do not exist. It is therefore necessary to further

BEST AVAILABLE COPY

characterize mesenchymal stem cells to identify probes or combinations of probes that can unequivocally identify mesenchymal stem cells in tissue. Such markers will also be useful for the isolation of mesenchymal stem cells from tissues.

Approximately one cell out of 100,000 nucleated cells in bone marrow aspirates is expected to be a mesenchymal stem cell. Currently, the main method for the isolation of mesenchymal stem cells from bone marrow is based on their capacity to adhere to plastic culture dishes and form colonies while the majority of bone marrow cells do not adhere and form colonies. These colonies are then further expanded and then induced with defined factors to differentiate into specific mesenchymal tissues. It is not clear, however, whether the mesenchymal stem cells isolated this way are a homogenous population. It will therefore be important to find markers that can be used to identify subclasses of mesenchymal stem cells with specific differentiation potentials.

In US 6 200 606, the isolation of cartilage or bone precursor cells from hematopoietic and non-hematopoietic cells by the use of CD34 as a marker and the further use of isolated stem cells in bone and cartilage regeneration processes is described. Still, no specific marker for mesenchymal stem cells is identified nor disclosed. The CD34 marker is expressed on early lymphohematopoietic stem and progenitor cells, small-vessel endothelial cells, embryonic fibroblasts, and some cells in fetal and adult nervous tissue, hematopoietic progenitors derived from fetal yolk sac, embryonic liver, and extra-hepatic embryonic tissues including aorta-associated hematopoietic progenitors in the 5 week human embryo.

Pittenger et al. ((1999) Science 284:143-147) have used a density centrifugation of human bone marrow to isolate human MSC. Cellular markers used to identify the MSC are SH-2, SH-3, CD29, CD44, CD71, CD90, CD106, CD120a, CD124.

Majumdar et al., ((2000) J. Cell. Physiol. 185:98-106) have used CD105 as a marker for enrichment of human MSC from bone marrow.

30 Denni et al., ((2002) Cells Tissues Organs 170:73-82) have used a marker called Stro-1 to enrich human MSC from bone marrow.

All markers mentioned so far may be used for enrichment of hMSC. Still, they are not exclusive for MSC, and it is unclear how homogenous the isolated MSC populations are when enriched using these markers. Monospecific and unique probes for the identification of hMSC do not exist as of today.

35 Furthermore, markers are needed to monitor the differentiation of mesenchymal stem cells into specific types of mesenchymal cells. This will be especially important when these cells are re-introduced into the human body to replace loss of damaged mesenchymal tissue, such as bone or cartilage.

BEST AVAILABLE COPY

Finally, the identification of specific cell surface markers for mesenchymal stem cells may be used for their isolation out of a complex mixture of cells by cell sorting techniques such as fluorescence activated cell sorting (FACS).

It is thus highly desirable in the light of the aforementioned problems to
5 identify and isolate MSC, for further use in bone, cartilage, muscle, bone marrow, tendon or connective tissue repair in vivo or in vitro. In this respect, the present invention addresses this needs and interest.

SUMMARY OF THE INVENTION

10 In view of the foregoing disadvantages known in the art when trying to isolate and identify mammalian MSC, the present invention provides marker for mammalian MSC suitable for identifying and isolating mammalian MSC.

One object with the present invention is to provide methods for identifying, or isolating mammalian MSC, or an enriched cellular population of MSC.

15 Another object is to provide uses of the marker according to the invention for identifying or isolating mammalian MSC.

Thus, the present invention provides a marker for mammalian mesenchymal stem cells. The marker comprises an integrin alpha 10 chain and/or integrin alpha 11 chain expressed on the cell surface of mesenchymal stem cell or intracellular in a
20 mesenchymal stem cell.

Further embodiments include wherein the integrin alpha 10 and/or integrin alpha 11 chain is expressed as a heterodimer in combination with an integrin beta 1 chain.

Also, the present invention provides a method for identifying a mammalian
25 mesenchymal stem cell. Such a method comprises the steps of

- a) providing a sample comprising a mesenchymal stem cell,
- b) detecting integrin alpha 10 and/or alpha 11 chain expression on the cell surface of a mesenchymal stem cell or intracellular in a mesenchymal stem cell,
- 30 c) scoring the integrin alpha 10 and/or alpha 11 chain expression, and
- d) identifying the mesenchymal stem cell according to the scoring in c) above.

Further embodiments include wherein the expression in b) above is detected by detecting the integrin alpha 10 and/or integrin alpha 11 protein expression.

35 Even further embodiments include wherein the expression in b) above is detected by detecting the integrin alpha 10 and/or integrin alpha 11 mRNA expression.

BEST AVAILABLE COPY

Even further, the present invention provides a method for determining whether a test compound modulates a mammalian mesenchymal stem cell differentiation.

5 Such a method comprises the steps of

- a) providing a mesenchymal stem cell
- b) contacting the mesenchymal stem cell with a test compound, and
- c) detecting a change in rate or pattern of differentiation of the mesenchymal stem cell as an indication of that the test compound modulates mesenchymal stem cell differentiation.

10

Still even further, the present invention provides a method for producing an isolated population of mammalian cells enriched for mesenchymal stem cells relative a reference population. Such a method comprises the steps of

15

- a) providing at least a portion of a population of cells, or a portion of a reference population, comprising MSC and at least one cell other than the mesenchymal stem cells,
- b) introducing into the population of cells in a) above a compound identifying the mesenchymal stem cells,
- c) selecting and isolating from the population of cells in b) above the mesenchymal stem cells, thereby producing a population of cells enriched for mesenchymal stem cells.

20

The method according to the invention may in further embodiments include wherein the mesenchymal stem cells is identified as a mesenchymal stem cell by detecting expression of integrin alpha 10 and/or alpha 11 chain expression on the cell surface of said mesenchymal stem cells according to the method disclosed in the present invention.

25

Even further, an enriched mammalian cellular population of mesenchymal stem cells, comprising at least one intact, viable mesenchymal stem cell is disclosed. Such enriched cellular population is a population wherein the mesenchymal stem cell is characterised by

30

- a) expressing an integrin alpha 10 chain and/or integrin alpha 11 chain on the cell surface of or intracellular in said mesenchymal stem cell,
- b) being substantially free from expression of molecules specific for committed lymphohematopoietic cells or uncommitted stem cells.

35

Also, an isolated mammalian mesenchymal stem cell expressing a marker according to the invention, obtainable by the method for producing a population of cells enriched for mesenchymal stem cells according to the invention is disclosed.

BEST AVAILABLE COPY

Still even further, a mammalian cellular composition comprising the enriched cellular population according to the invention, or the isolated mesenchymal stem cell according to the invention is disclosed.

- Uses of a marker according to the invention for identification of a
 5 mammalian mesenchymal stem cell, for modulating differentiation of a mammalian mesenchymal stem cell and for isolating a mammalian mesenchymal stem cell are also provided.

SHORT DESCRIPTION OF DRAWINGS

- 10 Fig. 1 shows a schematic view of a suggested stepwise transition from putative mesenchymal stem cell (MSC) to highly differentiated phenotypes. (From Caplan A.I. and Bruder S.P. Trends Mol. Med. 2001, 7(6): 259-264), and

- Fig. 2 shows that human mesenchymal stem cells in culture express both
 15 integrin alpha10 and alpha11 chains on their cell surface. In the figure, the upper band in both lanes is alpha10 (in the left lane) and alpha11 (in the right lane). The lower band in both lanes represent the beta1 chain.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

- 20 As used herein, the terms "rodent" and "rodents" refer to all members of the phylogenetic order *Rodentia*.

The term "murine" refers to any and all members of the family *Muridae*, including rats and mice.

- The term "substantially free from" is herein intended to mean at a below
 25 detection limits of the assay used thereby appearing negative, i.e. free from.

- The term "committed" is herein intended to mean dedicated to, or focused on. Thus, a committed cell is a cell that is dedicated to, or focused on a specific differentiation pathway. From this it will follow that an uncommitted cell is not dedicated to, or focused on, any specific differentiation pathway and has several
 30 options.

Integrin alpha10 and integrin alpha11 as a marker for MSC

- We have surprisingly found that the integrins alpha10beta1 and alpha11beta1
 35 are present on human mesenchymal stem cells. Thus, these integrins can be used to identify, differentiate, and isolate mesenchymal stem cells from a mixed cell population and will be a useful tool in cell therapy to repair damaged tissue.

The human integrin alpha10 chain is known and publicly available at GenBank™/EBI Data Bank accession number AF074015. Thus, new uses and methods of the integrin alpha10 chain are disclosed in the present invention.

BEST AVAILABLE COPY

The human integrin alpha 1 chain is known and publicly available at GenBank™/EBI Data Bank accession number AF137378. Thus, new uses and methods of the integrin alpha 1 chain are disclosed in the present invention.

As revealed above, the present invention relates to a marker for mesenchymal stem cells (MSC), comprising an integrin alpha 10 chain and/or integrin alpha 11 chain expressed on the cell surface of mammalian MSC or intracellular in mammalian MSC.

In a further embodiment, the integrin alpha 10 and/or integrin alpha 11 chain is expressed as a heterodimer in combination with an integrin beta 1 chain.

Mammalian MSC is generally isolated from bone marrow, peripheral blood, cord blood, liver, bone, cartilage, perichondrium, bone, periosteum or fat. The isolation may be based on the cells capacity to adhere to plastic culture dishes and form colonies under specific culture conditions, while the majority of bone marrow cells do not adhere and form colonies.

The colonies may further be expanded and then induced with defined factors to differentiate into specific mesenchymal tissues. For chondrocytes, the culture is a culture in pelleted micromass or in alginate without serum, and with TGFbeta3 added as a defined factor. For osteogenic cells, cells may be cultured in the presence of dexamethasone, beta-glycerol phosphate, ascorbate, and 10% FBS (foetal bovine serum), and for adipocytes, cells may be cultured in the presence of 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin. It is not clear, however, whether the mesenchymal stem cells isolated and expanded this way are a homogenous population.

Human MSC may be isolated from bone marrow, peripheral blood, cord blood, liver, bone, cartilage, perichondrium, and periosteum. The MSC may then further be isolated following a density centrifugation and found as a part of a mononuclear cell fraction layer at the density interface of 1.073 g/ml (Percoll™, Pharmacia). Out of this mononuclear cell fraction, 1/10 000 – 1/100 000 cells form colonies upon culture in serum in culture dishes.

Thus, the marker according to the invention, comprising an integrin alpha 10 chain and/or integrin alpha 11 chain, will be highly valuable for further evaluation and enrichment of the MSC population.

A method for identifying MSC

According to the invention, a method for identifying a mammalian MSC is disclosed. The method comprises the steps of

- a) providing a sample comprising MSC,
- b) detecting integrin chain alpha 10 and/or alpha 11 expression on the cell surface of a MSC or intracellular in MSC,

BEST AVAILABLE COPY

- c) scoring the integrin alpha10 and/or alpha11 chain expression, and
d) identifying the MSC according to the scoring in c) above.

The sample comprising mammalian MSC may be isolated from bone marrow, peripheral blood, cord blood, liver, bone, cartilage, perichondrium, and periosteum. As described in the paragraph above, the MSC may be isolated by plastic adhesion of a mixed cell population, followed by further optional expansion of the cells with defined factors to differentiate into different mesenchymal tissues. For chondrocytes, the culture may be a culture in pelleted micromass or in alginate with or without serum, and with TGFbeta3 added as a defined factor. For osteogenic cells, cells may be cultured in the presence of dexamethasone, beta-glycerol phosphate, ascorbate, and 10% FBS (foetal bovine serum), and for adipocytes, cells may be cultured in the presence of 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin

In further embodiments of the invention, other markers may be analysed in parallel with the marker according to the invention. Such other markers are SH-2, SH-3, CD29, CD44, CD71, CD90, CD106, CD120a, CD124, CD105, and Stro-1 that MSC may express. Markers that do not express on MSC are CD14, CD34 and CD45 and their expression may in further embodiments also be evaluated together with the marker according to the invention.

In a further embodiment, the expression in b) above is detected by detecting the integrin alpha10 and/or integrin alpha 11 protein expression.

In still a further embodiment, the integrin chain alpha10 and/or alpha 11 expression is detected on the cell surface of a MSC or intracellular in a MSC.

In still a further embodiment, the expression in b) above is detected by an immunoassay. The detection may be performed by various methods, e.g. any immunomethod known to the skilled man in the art, such as immunoprecipitation, Western blotting or flow cytometry methods, e.g. fluorescence activated cell sorting (FACS). Monoclonal antibodies are particularly useful for identifying markers, surface membrane proteins as well as intracellular markers, associated with particular cell lineages and/or stages of differentiation. Thus, it is suitable for the identification of integrin alpha10 as well as alpha 11. Still, identification may as well be performed by any specific molecule, such as a protein or peptide, binding specifically to the integrin alpha10 and/or the integrin alpha 11 molecule. Examples of such proteins or peptides are natural ligands, binding to the integrin alpha10 and/or the integrin alpha 11 molecule. Such natural ligands may be made recombinant, chemically synthesised, or purified from a natural source.

In still a further embodiment, the expression above is detected by detecting the the integrin alpha10 and/or integrin alpha 11 mRNA expression. Detection of

BEST AVAILABLE COPY

2002-03-14

9

Huvudföreläsning

mRNA expression of a specific protein is well known to the skilled man in the art, and is generally done by probing the mRNA with a DNA or RNA probe specific for the mRNA of interest, under hybridisation conditions where the probe is not hybridising to other mRNA molecules. Different polymerase chain reactions (PCR)

5 may also be used, which is obvious to the skilled man in the art.

The scoring of the integrin alpha10 and/or the integrin alpha11 molecule expression may be done relative to a reference cell population expressing the integrin alpha10 and/or the integrin alpha11 molecule, as well as not expressing the integrin alpha10 and/or the integrin alpha11 molecule.

10 The subsequent identification of MSC is based upon the above described scoring.

A method for producing an isolated population of cells enriched for mammalian MSC

15 According to the invention, a method is disclosed for producing an isolated population of cells enriched for mammalian MSC relative a reference population, the method comprising the steps of

- 20
- a) providing a at least a portion of a population of cells, or at least a portion of a reference population, comprising MSC and at least one cell other than a MSC,
 - b) introducing into the population of cells in a) above a compound identifying the MSC,
 - c) selecting and isolating from the population of cells in b) above the MSC, thereby producing a population of cells enriched for MSC.

25 Providing a population is described in the paragraphs above, and may be performed in a similar way as in the method for identification of MSC. If the population of cells is collected from BM, at about 0.01-0.001% of the starting population, or "crude population", is MSC. Though, this may vary between different donors.

30 The compound introduced to identify the MSC may be a protein, peptide, monoclonal antibody, or part thereof, or polyclonal antibody identifying the MSC. In one embodiment, the MSC is identified as a MSC by detecting expression of integrin chain alpha10 and/or alpha11 expression on the cell surface of said MSC according to the method for identifying MSC described above.

35 The selection and isolation of MSC is a separation step for separating the identified MSC. Various techniques may be employed to separate the cells by initially removing cells dedicated to other lineages than MSC. Monoclonal or polyclonal antibodies, or parts thereof, are particularly useful for identifying markers, here on intact viable cells being surface membrane proteins, associated

BEST AVAILABLE COPY

with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to allow for a first crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed known to the skilled man in the art. The particular technique employed will depend upon efficiency of separation, cytotoxicity of the methodology, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation may include magnetic separation, using e.g. antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, e.g., complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., a plate, or other convenient techniques. Techniques providing accurate separation include fluorescence activated cell sorters, which can have varying degrees of sophistication, e.g., a plurality of colour channels, light scattering detecting channels, impedance channels, etc. known to the skilled man in the art.

In one embodiment, the first enrichment step of MSC may be a negative selection of the MSC, i.e. other lineage committed cells are depleted, or removed, from the initial population of cells.

In still a further embodiment, the first enrichment is a positive selection of MSC that may be repeated till the desired purity of the MSC is achieved. For a positive or a negative selection, proteins, peptides, monoclonal or polyclonal antibodies may be used as a compound to identify the integrin alpha10 or integrin alpha11 molecule as described above. The compound may be conjugated with means for separation, such as magnetic beads, which allow for direct separation; biotin, which can be removed with avidin; or streptavidin bound to a support; fluorochromes, which can be used with a fluorescence activated cell sorter; or the like, to allow for ease of separation of the particular cell type. Any technique may be employed which is not unduly detrimental to the viability of the cells of interest, i.e. the MSC.

In one embodiment, the selection is performed by fluorescent cell sorting, by using e.g. a fluorescence activated cell sorter (FACS) or any other methodology having high specificity. Multi-color analyses may be employed with the FACS which is particularly convenient. The cells may be separated on the basis of the level of staining for the particular antigens. In a first separation, antibodies for other markers may be used labeled with one fluorochrome, while the antibodies for the dedicated lineages, i.e. the integrin alpha10 and/or integrin alpha11, may be conjugated to (a) different fluorochrome(s). Other markers may in further embodiments be SH-2, SH-3, CD29, CD44, CD71, CD90, CD106, CD120a, CD124, CD105, and Stro-1 that MSC may express. Markers that do not express on

BEST AVAILABLE COPY

MSC are CD14, CD34 and CD45 and their expression, or lack of, may in further embodiments also be evaluated together with the marker according to the invention.

If further lineages or cell populations are to be removed in this step, various antibodies to such lineage specific markers may be included. Fluorochromes which may find use in a multi-color analysis include phycobilliproteins, e.g., phycoerythrin and allophycocyanins, fluorescein, Texas red, etc.

The cells may be selected against dead cells, by employing dyes associated with dead cells (propidium iodide, LDS). The cells may be collected in a medium comprising fetal calf serum.

Other techniques for positive or negative selection may be employed, which permit accurate separation, such as affinity columns, and the like.

Cells may be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a crude separation, preferably a negative selection, followed by a fine separation, which is a positive selection, with positive selection of a marker associated with MSC and negative selection for markers associated with lineage committed cells, and other stem cell populations not being MSC. This separation is followed by selection for a cellular composition having multi-lineage potential as a MSC and enhanced self-regeneration capability. The composition is further described below.

Isolated mammalian MSC

According to the invention, an enriched cellular population of mammalian MSC is disclosed. Such a cellular population comprises intact, viable MSC, wherein the MSC are characterised by

- a) expressing an integrin alpha 10 chain and/or integrin alpha 11 chain on the cell surface of said MSC or intracellular in MSC,
- b) being substantially free from expression of molecules specific for committed hematopoietic cells.

Molecules specific for committed hematopoietic cells are e.g. CD45.

Other molecules the MSC cells are substantially free from are e.g. CD34 and CD14.

In further embodiments, the enrichment of such a population is about 70, 80, 90, 95, 98, 99, 99.9, or even 100%.

According to the invention an isolated MSC expressing a marker according to the invention is disclosed. The isolated MSC are obtainable by the method for producing a population of cells enriched for MSC according to the invention.

BEST AVAILABLE COPY

BEST AVAILABLE COPY

5

10

15

20

30

35

Modulation of MSC

According to the invention a method for determining whether a test compound modulates a mammalian MSC differentiation is disclosed. Such a method comprises the steps of

- 5 a) providing a MSC
 b) contacting the MSC with a test compound, and
 c) detecting a change in rate or pattern of differentiation of the MSC as an indication that the test compound modulates MSC differentiation.

10 The MSC provided may be an enriched cell population achieved according to any of the methods disclosed, the isolated MSC according to the invention, or the cellular composition according to the invention.

 The test compound may be any compound known to affect or suspected to affect MSC, e.g. pharmaceutical compositions, drugs, polyclonal or monoclonal antibodies, or parts thereof, such as antibodies binding to integrin alpha10 and/or
15 integrin alpha11 or any other molecule on the MSC, factors used to promote growth of MSC, e.g. FBS, FGF, or factors used to promote differentiation of MSC, e.g. dexamthasone, TGFbeta, insulin.

 The detection of a change in rate or pattern of differentiation of the MSC as an indication that the test compound modulates MSC differentiation may be done
20 via flow cytometry or any other suitable method, such as any immunomethod, known to the skilled man in the art. The change in rate or pattern of differentiation may be kinetical, functional or phenotypical studies of the MSC modulated with the test compound, relative for an untreated, or mock treated, MSC population. It may also be a comparison relative to least one second test compound.

25 In a further embodiment the MSC is identified as a MSC by detecting expression of integrin chain alpha10 and/or alpha11 expression on the cell surface of said MSC or intracellular in MSC according to the method of identifying MSC disclosed herein.

30 *Use of a marker*

 The mammalian MSC, such as human or mouse, provided herein find a number of uses. For instance, 1) re-generation of a host deficient in MSC; 2) treatment of a host by the re-engraftment of MSC for re-generation of bone, cartilage, muscle, marrow, tendon/ligament and connective tissue in a patient in the
35 need thereof; 3) in detecting and evaluating growth factors relevant to MSC self re-generation ; 4) in development of MSC lineages and screening for factors associated with their development and differentiation.

 Further, the marker according to the invention has several uses. The marker according to the invention comprises the integrin alpha10 and/or alpha11 chain

expression. Said marker may thus be used to identify, differentiate, and isolate mammalian mesenchymal stem cells from a mixed cell population and will be a useful tool in cell therapy to repair damaged tissue.

According to the invention, use of the marker according to the invention is disclosed for identification of MSC.

Further, a use of a marker according to the invention is disclosed, for modulating differentiation of a MSC.

Still even further, a use of a marker according to the invention is disclosed, for isolating a MSC or an enriched population of MSC.

Mammalian MSC

In the methods and uses disclosed in the present invention, mammalian MSC, mammalian cellular populations and mammalian cellular compositions are disclosed.

In specific embodiments, the mammal may be a human.

Still further embodiments include wherein the mammal is a rodent, such as a rat, mouse, or any other member of the family Muridae.

EXAMPLES

Example 1 Detection of integrin alpha10 and integrin alpha11 chain on human MSC

Objective

The objective of this example is to analyse human MSC for the expression of integrin alpha10 and alpha11, using immunoprecipitation.

Materials and methods

Human mesenchymal stem cells (obtained from In Vitro, Sweden, at passage 2), were cultured in MSCBM medium (provided by In Vitro, Sweden) until passage 4 and then surface biotinylated.

In brief, cells adherent on the plate were washed once with PBS and then surface biotinylated using 0.5mg/ml Sulfo-NHS-LC-biotin (Pierce) in 4ml PBS for 20min. Cells were then washed once with PBS and 10ml 0.1M glycine/PBS were added for 5min.

After washing once with PBS cells were lysed in 1ml lysis buffer (1% NP40, 10% glycerol, 20mM Tris/HCl, 150mM NaCl, 1mM MgCl₂, 1mM CaCl₂, protease inhibitor cocktail BM, pH7.5). The cell lysate was collected with a plastic scraper, pipetted into an eppendorf tube and spun down for 10min at 15.000g.

BEST AVAILABLE COPY

The supernatant collected from the centrifugation step above was incubated with 2microliter of alpha10 pre-immune serum followed by addition of 20µl Prot G sepharose (Amersham) in 100µl lysis buffer.

After rotating the cells in lysis buffer over night at 4°C the lysate was
5 centrifuged for 1min at 8000 rpm and the supernatant removed. For each
immunoprecipitation 150µl cell lysate were pipetted into an eppendorf tube and 1µl
of antiserum was added. The sera used were rabbit-anti-human α10 and rabbit-anti-
human α11, respectively (both sera against the cytoplasmic domains of the
integrins).

10 After 1h rotating at 4°C, 20µl protein G Sepharose (Amersham) in 100µl lysis buffer was added and the mixture further rotated for another 30min.

The Sepharose-beads were then spun down briefly and washed three times with lysis buffer.

20µl SDS-PAGE sample buffer (including 100mM DTT) was added to the
15 Sepharose beads and then the samples were boiled for 5min. 5µl of each sample
were run on a 8% SDS-PAGE gel (Novex) and then electro-transferred onto a
PVDF membrane.

The membrane was blocked in 2% BSA/TBST (TBST: 20mM Tris/HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1h, washed once with TBST and then
20 incubated with 2 μ l Extravidin-peroxidase (Sigma) in 8ml blocking buffer.

After 1h the Extravidin-peroxidase solution was removed and the membrane washed 3x20min in TBST. Surface biotinylated proteins were then detected with ECL (Amersham) and visualised on a photographic film.

25 Results and discussion

In figure 2, the results of the immunoprecipitation is shown. Human mesenchymal stem cells in culture express both integrins alpha10 and alpha11 on their surface. In the figure, the upper band in both lanes is alpha10 (in the left lane) and alpha11 (in the right lane). The lower band in both lanes represent the beta1 chain.

Both integrin alpha10 and alpha11 expression is identified.

CLAIMS

- 1.** A marker for mammalian mesenchymal stem cells, comprising an integrin alpha 10 chain and/or integrin alpha 11 chain expressed on the cell surface of a mesenchymal stem cell or intracellular in a mesenchymal stem cell.
- 2.** The marker according to claim 1, wherein the integrin alpha10 and/or integrin alpha 11 is expressed as a heterodimer in combination with an integrin beta1 chain.
- 3.** A method for identifying a mammalian mesenchymal stem cell, the method comprising the steps of
- a) providing a sample comprising a mesenchymal stem cell,
 - b) detecting integrin chain alpha10 and/or alpha11 expression on the cell surface of a mesenchymal stem cell or intracellular in a mesenchymal stem cell,
 - c) scoring the integrin chain alpha10 and/or alpha11 expression, and
 - d) identifying the mesenchymal stem cell according to the scoring in c) above.
- 4.** The method according to claim 3, wherein the expression in b) above is detected by detecting the integrin alpha10 and/or integrin alpha 11 protein expression.
- 5.** The method according to claim 3, wherein the expression in b) above is detected by detecting the integrin alpha10 and/or integrin alpha 11 mRNA expression.
- 6.** The method according to any of claims 3-4, wherein the expression in b) above is detected by an immunoassay.
- 7.** A method for determining whether a test compound modulates a mammalian mesenchymal stem cell differentiation, the method comprising the steps of
- a) providing a mesenchymal stem cell
 - b) contacting the mesenchymal stem cell with a test compound, and
 - c) detecting a change in rate or pattern of differentiation of the mesenchymal stem cell as an indication of that the test compound modulates a mesenchymal stem cell differentiation.

8. The method according to claim 7, wherein the rate or pattern of differentiation is detected by detecting integrin chain alpha10 and/or alpha11 expression on the cell surface of said mesenchymal stem cell or intracellular in a mesenchymal stem cell according to the method in any of claims 3-6.
9. A method for producing an isolated population of mammalian cells enriched for mesenchymal stem cells relative a reference population, the method comprising the steps of
- a) providing at least a portion of a population of cells, or a portion of a reference population, comprising a mesenchymal stem cell and at least one cell other than a mesenchymal stem cells,
 - b) introducing into the population of cells in a) above a compound identifying the mesenchymal stem cells,
 - c) selecting and isolating from the population of cells in b) above the mesenchymal stem cells, thereby producing a population of cells enriched for mesenchymal stem cells.
10. The method according to claim 9, wherein the mesenchymal stem cells is identified as a mesenchymal stem cell by detecting expression of integrin alpha10 and/or alpha11 chain expression on the cell surface of said mesenchymal stem cells according to the method in any of claims 3-6.
11. The method according to any of claims 9-10, wherein the selection in c) above is performed by fluorescent cell sorting.
12. An enriched mammalian cellular population of mesenchymal stem cells, comprising at least one intact, viable mesenchymal stem cell, wherein the mesenchymal stem cell are characterised by
- a) expressing an integrin alpha 10 chain and/or integrin alpha 11 chain on the cell surface of or intracellular in said mesenchymal stem cell,
 - b) being substantially free from expression of molecules specific for committed lymphohematopoietic cells or uncommitted stem cells.
13. An isolated mammalian mesenchymal stem cell expressing a marker according to any of claims 1-2, obtainable by the method for producing a population of cells enriched for mesenchymal stem cells according to any of claims 9-10.

BEST AVAILABLE COPY

14. A mammalian cellular composition comprising the enriched cellular population according to claim 12, or the isolated mesenchymal stem cell according to claim 13.
- 5 15. Use of a marker according to any of claims 1-2, for identification of a mammalian mesenchymal stem cell.
16. Use of a marker according to any of claims 1-2, for modulating differentiation of a mammalian mesenchymal stem cell.
- 10 17. Use of a marker according to any of claims 1-2, for isolating a mammalian mesenchymal stem cell.

BEST AVAILABLE COPY

0001001-15

ABSTRACT

A marker for mesenchymal stem cells (MSC) is provided, comprising an integrin alpha10 chain and/or an integrin alpha11 chain expressed on the cell surface of or intracellular in a MSC. The marker is used in methods for isolation of
5 mammalian MSC and in methods for identification of MSC. Also included are isolated cellular populations of mammalian MSC and a cellular composition comprising the latter. Moreover, uses of said marker for isolation, modulation and identification of mammalian MSC are provided.

BEST AVAILABLE COPY

0201831-15



Figure 1

+46 40 6119660

Ex. 1. First set of vowels

14

Hoover-Allen Kesson

BEST AVAILABLE COPY



Figure 2